In the specification

On page 1, in the blank space at line 5 above the heading "Background of the Invention", please insert the following paragraph::

This application is a divisional of U.S.S.N. 09/763,620 filed March 2, 2001 entitled "*Attenuated Salmonella SP12 Mutants as Antigen Carriers*", which is a filing under 35 USC §371 of PCT/EP99/06514 (WO 00/14240) filed on September 3, 1999, which claims priority to European Patent Application No. 98116827.1 filed September 4, 1998.

Please replace the paragraph on page 35, lines 3-18 with the following paragraph:

Genomic DNA of various *Salmonella* strains and *E. coli* K-12 was prepared as previously described (Hensel *et al.*, 1997a). For Southern hybridization analysis, genomic DNA was digested with *Eco*RI or *Eco*RV, fractionated on 0.6 % agarose gels and transferred to Hybond N^+ membranes (Amersham, Braunschweig). Various probes corresponding to the SPI-2 region were obtained as restriction fragments of the subcloned insert of λ1. Probes corresponding to ORF 242 and ORF 319 were generated by PCR using primer sets D89 (5'-TTTTTACGTGAAGCGGGGTG-3') (SEQ ID NO:44) and D90 (5'-GGCATTAGCGGATGTCTGACTG-3') (SEQ ID NO:45), and D91 (5'- CACCAGGAACCATTTTCTCTGG-3') (SEQ ID NO:46) and D92 (5'-CAGCGATGACGATATTCGACAAG-3') (SEQ ID NO:47), respectively. PCR was performed according to the specifications of the manufacturer (Perkin-Elmer, Weiterstadt). PCR products were submitted to agarose gel electrophoresis and fragments of the expected size were

recovered and purified. Hybridization probes were labeled using the DIG labeling system as described by the manufacturer (Boehringer, Mannheim).

Please replace the paragraph bridging pages 42 and 43 with the following paragraph:

- Mutant MvP103, *sseC*. A 2.6kb fragment was recovered after *Bam*HI and *Cla*I digestion of p5-2 and subcloned in *Bam*HI/*Cla*I-digested pBluescript II KS+. The resulting construct termed p5-50 was digested by *Hin*dIII, blunt ended using the Klenow fragment of DNA polymerase and ligated to the *aphT* cassette. A 900 bp *Hinc*II fragment of pSB315 containing an aminoglycoside 3'-phosphotransferase gene (*aphT*) from which the transcriptional terminator had been removed (Galán *et al.*, 1992) was ligated in the same orientation into the blunted-ended *Hin*dIII site of plasmid p5-50. After transformation of *E. coli* XL-1 Blue and selection for resistance against kanamycin and carbenicillin (50 µg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p5-51 and its identity confirmed by restriction analysis. It was further digested with *Sal*I and *Xba*I and the insert of 3.5kb was ligated to *Sal*I/*Xba*I-digested pGP704. This plasmid was electroporated into *E. coli* CC118 λ*pir* and the transformants selected for resistance to kanamycin and carbenicillin (50 ìg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pGP704, termed p5-53, isolated and confirmed by restriction analysis. Plasmid p5-53 was electroporated into *E. coli* S17-1 λ*pir* and transferred into *S. typhimurium* NCTC12023 (resistant to nalidixic acid, 100 µg/ml) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *sseC* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected

by resistance to kanamycin and nalidixic acid (100 μg/ml). The resulting exconjugants were finally tested for a lactose-negative phenotype and their sensitivity to carbenicillin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which have been acquired during the cloning procedure the mutated *sseC* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP103 was examined for the presence of the resistance cassette within the *sseC* gene by the use of PCR. Amplification was performed by using the primers E25 (5'-GAAATCCCGCAGAAATG-3') (SEQ ID NO:48) and E28 (5'-AAGGCGATAATATAAAC-3') (SEQ ID NO:49). The resulting fragment had a size of 1.6kb for *S. typhimurium* wild-type and 2.5kb for strain MvP103.

Please replace the paragraph bridging pages 44 and 45 with the following paragraph:

- Mutant MvP101, *sseD.* A 3.0kb fragment was recovered after *Pst*I and *Eco*RI digestion of p5-2 and subcloned in *PstI/Eco*RI-digested pUC18 The resulting construct termed p5-30 was digested by *Eco*RV and treated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p5-30 in the same orientation in the unique *Eco*RV site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 μg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p5-31 and its identity confirmed by restriction analysis. p5-31 was further digested with *Sph*I and *Eco*RI, a 4.0kb fragment isolated and ligated to *SphI/Eco*RI-digested pGP704. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformants selected to

kanamycin and carbenicillin (50 μg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pGP704, termed p5-33, isolated and confirmed by restriction analysis. Plasmid p5-33 was electroporated into E. coli S17-1 λ*pir* and transferred into *S. typhimurium* NCTC12023 (resistant to nalidixic acid) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *sseD* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by resistance to kanamycin and nalidixic acid (100 μg/ml). The resulting exconjugants were finally tested for a lactose-negative phenotype and their sensitivity to carbenicillin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which might have been accumulated during the cloning procedure the mutated *sseD* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP101 was examined for the presence of the resistance cassette within the *sseD* gene by the use of PCR. Amplification was performed by using the primers E6 (5'-AGAGATGTATTAGATAC-3') (SEQ ID NO:50) and E28 (5'-AAGGCGATAATATAAAC-3') (SEQ ID NO:49). The resulting fragment had a size of 0.8kb for *S. typhimurium* wild-type was used and 1.7kb in the case of strain MvP101.

Please replace the paragraph bridging pages 45 and 46 with the following paragraph:

- Mutant MvP102, deletion of parts of *sseE* and *sscB*. A 4.5kb fragment was recovered after *Sst*I and *Hin*dIII digestion of p5-2 and subcloned in *Sst*I/*Hin*dIII-digested pKS+. The resulting construct termed p5-40 was digested by *Sma*I, digested with alkaline phospatase and ligated to the

aphT cassette in the same orientation into the unique *Sma*I site created in the *sseE/sseB* deletion plasmid p5-40 as described above. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 µg/ml each) one clone was chosen and the harbouring plasmid isolated. This plasmid was termed p5-41 and its identity confirmed via restriction analysis. It was further digested with *Kpn*I and *Sst*I and the insert was ligated to *Kpn*I/*Sst*I-digested pNQ705. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformed bacteria selected to kanamycin and chloramphenicol (50 µg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pNQ705, termed p5-43, isolated and confirmed by restriction analysis. The resulting plasmid was used to transfer the mutated gene onto the *Salmonella* chromosome as described above. Resulting clones have been further examined by Southernblot analysis. To exclude possible mutations which might have been acquired during the cloning procedure the mutated *sseE/sscB* allele was transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP102 was examined for the presence of the resistance cassette within the *sseE/sseB* gene by the use of PCR. Amplification was performed by using the primers E6 (5'-AGAGATGTATTAGATAC-3') (SEQ ID NO:50) and E4 (5'-GCAATAAGAGTATCAAC-3') (SEQ ID NO:51). The resulting fragment had a size of 1.6kb for *S. typhimurium* wild-type and a size of 1.9kb for strain MvP102.

Please replace the paragraph bridging pages 47 and 48 with the following paragraph:

A deletion of 158 bp between codon 264 and 422 of *sseC* was generated. Plasmid p5-2 was digested by *Cla*I and the larger fragment containing the vector portion was recovered and self-

ligated to generate p5-60. Plasmid p5-60 was linearized by digestion with *Hin*dIII, which cuts once within the *sseC* gene. Primers *sseC*-del-1 (5'- GCT AAG CTT CGG CTC AAA TTG TTT GGA AAA C -3') (SEQ ID NO:52) and *sseE*-del-2 (5'- GCT AAG CTT AGA GAT GTA TTA GAT ACC -3') (SEQ ID NO:53) were designed to introduce *Hin*dIII sites. PCR was performed using linearized p5-60 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Hin*dIII, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction digestion and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *XbaI*/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λpir to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots

were plated on LB containing 250 μg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sseC*-For (5'- ATT GGA TCC GCA AGC GTC CAG AA -3') (SEQ ID NO:54) and *sseC*-Rev (5- TAT GGA TCC TCA GAT TAA GCG CG-3') (SEQ ID NO:55). Amplification of DNA from clones containing the wild-type *sseC* allele resulted in a PCR product of 1520 bp, use of DNA from clones harbouring a *sseC* allele with an internal deletion resulted in a PCR product of 1050 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southern analysis of the sseC locus. Finally, the *sseC* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP 337.

Please replace the paragraph bridging pages 48 and 49 with the following paragraph:

A deletion of 116 bp between codon 26 and 142 of *sseD* was generated. Plasmid p5-2 was digested by *Hin*dIII/*Pst*I and a fragment of 2.1kb was isolated and subcloned in *Hin*dIII/*Pst*I-digested vector pBluescript SK+. The resulting construct was designated p5-8. p5-8 was linearized by digestion with *Eco*RV, which cuts twice within the *sseD* gene. Primers *sseD*-del-1 (5'- ATA GAA TTC GGA GGG AGA TGG AGT GGA AG -3') (SEQ ID NO:56) and *sseD*-del-2 (5'- ATA GAA TTC GAA GAT AAA GCG ATT GCC GAC -3') (SEQ ID NO:57) were designed to introduce *Eco*RI sites. PCR was performed using linearized p5-8 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 μl

volume were set up using 10 µl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 5 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Eco*RI, self-ligated, and the ligation mixture was used to transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction mapping and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 ìg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseD* locus was performed by PCR using primers *sseD*-For (5'- GAA GGA TCC ACT CCA TCT CCC TC -3') (SEQ ID NO:58) and *sseD*-Rev (5- GAA GGA TCC ATT TGC TCT ATT TCT TGC-3') (SEQ ID NO:59). Amplification

of DNA from clones containing the wild-type *sseD* allele resulted in a PCR product of 560 bp, use of DNA from clones harbouring a *sseD* allele with an internal deletion resulted in a PCR product of 220 bp. The integrity of clones harbouring the *sseD* deletion was further confirmed by Southernanalysis of the *sseD* locus. Finally, the *sseD* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP338.

Please replace the paragraph bridging pages 50 and 51 with the following paragraph:

A deletion of 128 bp between codon 32 and 160 of *ssc*B was generated. A 3kb *Bgl*II fragment of plasmid p5-2 was ligated into the *Bam*HI site of pBluescript KS+ to generate plasmid p5-70. Plasmid p5-70 was linearized by digestion with *Nco*I, which cuts once within the *sscB* gene. Primers *sscB*-del-1 (5'- ATG GGA TCC GAG ATT CGC CAG AAT GCG CAA -3') (SEQ ID NO:60) and *sscB*-del-2 (5'- ATG GGA TCC ACT GGC ATA AAC GGT TTC CGG -3') (SEQ ID NO:61) were designed to introduce *Bam*HI sites. PCR was performed using linearized p5-70 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-70), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Bam*HI, self-ligated, and the ligation mixture was used to

transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 ìg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sscB*-For (5'- ATT GGA TCC TGA CGT AAA TCA TTA TCA -3') (SEQ ID NO:62) and *sscB*-Rev (5- ATT GGA TCC TTA AGC AAT AAG TGA ATC -3') (SEQ ID NO:63). Amplification of DNA from clones containing the wild-type *sscB* allele resulted in a PCR product of 480 bp, use of DNA from clones harbouring a *sscB* allele with an internal deletion resulted in a PCR product of 100 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southernanalysis of the *sscB* locus. Finally, the *sscB* locus containing the internal in-frame deletion was moved into a fresh strain background of

S. typhimurium by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP339.

Please replace the paragraph bridging pages 51 and 52 with the following paragraph:

In a further approach the complete sequence of the chromosomal *sseC* gene was deleted by allelic replacement with a deleted copy of the gene. The deletion was constructed in a suicide plasmid (pCVD442 (Donnenberg et al., 1991). First, two DNA fragments flanking the *sseC* gene (fragment A, carrying artificial *SalI* and *XbaI* sites at its 5' and 3' ends, respectively; and fragment B, carrying artificial *XbaI* and *SacI* sites at its 5' and 3' ends, respectively) were amplified by PCR. The oligonucleotides used for PCR were: 1.) sseDelfor1 GCTGTCGACTTGTAGTGAGTGAGCAAG (SEQ ID NO:70) (3' nucleotide corresponds to bp 941 in included sequence: Fig 21A); 2.) sseCDelrev2 GGATCTAGATTTTAGCTCCTGTCAGAAAG (SEQ ID NO:71) (3' nucleotide corresponds to bp 2585 in included sequence, oligo binds to reverse strand); 3.) sseCDelfor2 GGATCTAGATCTGAGGATAAAAATATGG (SEQ ID NO:72) (3' nucleotide corresponds to bp 4078 in included sequence); 4.) sseDelrev1 GCTGAGCTCTGCCGCTGACGGAATATG (SEQ ID NO:73) (3' nucleotide corresponds to bp 5592 in included sequence, oligo binds to reverse strand). The resulting PCR fragments were fused together via the *XbaI* site. The resulting fragment was cut with *SalI* and *SacI* and cloned into pCVD442 cut with *SalI* and *SacI*. The resulting plasmid was introduced into *S.typhimurium* NCTC12023 by conjugation and chromosomal integrants of the plasmid into the *sseC* locus were selected for by the plasmid-encoded ampicillin resistance marker.

In a second step, clones which had lost the plasmid were screened for by loss of ampicillin resistance. The resulting clones were tested for chromosomal deletion of the *sseC* gene by PCR, and deletion of a 1455 bp fragment, comprising the entire *sseC* open reading frame, was confirmed. This Δ*sseC* mutant strain was named III-57Δ*sseC*.

Please replace the paragraph bridging pages 71 and 72 with the following paragraph:

- Mutant MvP284, *ssrA*. The *ssrA* gene (Fig. 12) was subcloned from the phage clone λ2 derived plasmid p2-2 on a 5.7kb *Bam*HI fragment in pUC18 as indicated in Table 1. A 1.6kb fragment was recovered after *Hin*dIII and *Eco*RV digestion of p2-2 and subcloned in *Hin*dIII/*Hin*cII-digested pBluescript II KS+. The resulting construct termed p2-20 was digested with *Hin*cII and dephosphorylated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p2-20 in the same orientation into the unique *Hin*cII site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 µg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p2-21 and its identity proved via restriction analysis. p2-21 was further digested with *Kpn*I and *Xba*I, a 2.5kb fragment isolated and ligated to *Kpn*I/*Xba*I-digested pKAS32. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformants selected to kanamycin and carbenicillin (50 µg/ml each). As done before, one clone was chosen, its plasmid with the according DNA fragment in pKAS32, termed p2-22, isolated and confirmed by restriction analysis. Plasmid p2-22 was electroporated into *E. coli* S17-1 λ*pir* and transferred into *S. typhimurium* NCTC12023 (streptomycin resistant) by conjugation as has been described previously (de Lorenzo

and Timmis, 1994). Exconjugants in which the *ssrA* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by its growth on M9+glucose minimal medium agar plates (Maloy *et al.*, 1996) and its resistance to kanamycin and carbenicillin (100 μg/ml). The resulting exconjugants were finally shown to have a lactose negative phenotype and to be sensitive to kanamycin and streptomycin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which might have been developed during the cloning procedure the mutated *ssrA* allele was transfered into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996). The resulting *Salmonella* strain MvP284 was examined for the presence of the resistance cassette within the *ssrA* gene by the use of primers *ssrA*-For (5'- AAG GAA TTC AAC AGG CAA CTG GAG G-3') (SEQ ID NO:64) and *ssrA*-Rev (5- CTG CCC TCG CGA AAA TTA AGA TAA TA -3') (SEQ ID NO:65). Amplification of DNA from clones containing the wild-type *ssrA* allele resulted in a PCR product of 2800 bp, use of DNA from clones harbouring a *ssrA* allele disrupted by the *aphT* cassette resulted in a PCR product of 3750bp. The resulting *Salmonella* strain MvP320 was examined for the presence of the resistance cassette within the *ssrB* gene by the use of Southern hybridization analysis of total DNA of exconjugants.

Please replace the paragraph bridging pages 72 and 73 with the following paragraph:

- Mutant MvP320, *ssrB*. The *ssrB* gene (Fig. 12) was subcloned from the phage clone λ1 derived plasmid p1-6 on a 4.8kb *Pst*I/*Bam*HI-fragment in pT7-Blue as indicated in Table 1. A 1.7kb fragment was recovered after *Bam*HI and *Hinc*II digestion of p1-6 and subcloned in

*Bam*HI/*Hin*cII-digested pBluescript II KS+. The resulting construct termed p1-20 was digested with *Eco*RV and dephosphorylated with alkaline phosphatase. The *aphT* cassette was isolated as described above and ligated to the linearized plasmid p1-20 in the same orientation into the unique *Eco*RV site. After transformation of *E. coli* XL-1 Blue and selection against kanamycin and carbenicillin (50 µg/ml each) one clone has been chosen and the harbouring plasmid isolated. This plasmid was termed p1-21 and its identity confirmed by restriction analysis. p1-21 was further digested with *Kpn*I and *Xba*I, a 2.5kb fragment isolated and ligated to *Kpn*I/*Xba*I-digested pKAS32. This plasmid was electroporated into *E. coli* CC118 λ*pir* and transformed bacteria selected to kanamycin and carbenicillin (50 µg/ml each) was performed. As done before, one clone was chosen, its plasmid with the according DNA fragment in pKAS32, termed p1-22, isolated and confirmed by restriction analysis. Plasmid p1-22 was electroporated into *E. coli* S17-1 λ*pir* and transferred into *S. typhimurium* NCTC12023 (streptomycin resistant) by conjugation as has been described previously (de Lorenzo and Timmis, 1994). Exconjugants in which the *ssrB* gene had been replaced by the cloned gene disrupted by insertion of the *aphT* cassette were selected by its growth on M9+glucose minimal medium agar plates (Maloy *et al.*, 1996) and its resistance to kanamycin and carbenicillin (100 µg/ml). The resulting exconjugants were finally shown to have a lactose negative phenotype and to be sensitive to kanamycin and streptomycin. Selected clones were further examined by Southernblot analysis. In order to exclude possible mutations which might have been acquired during the cloning procedure the mutated *ssrB* allele has been transferred into a fresh *Salmonella* background by P22 transduction (described by Maloy *et al.*, 1996).

Screening of mutants with a insertion of the *aphT* cassette within the *ssrB* locus was performed by PCR using primers *ssrB*-For (5'- CTT AAT TTT CGC GAG GG -3') (SEQ ID NO:66) and *ssrB*-Rev (5'- GGA CGC CCC TGG TTA ATA -3') (SEQ ID NO:67). Amplification of DNA from clones containing the wild-type *ssrB* allele resulted in a PCR product of 660 bp, use of DNA from clones harbouring a *ssrB* allele disrupted by insertion of the *aphT* cassette resulted in a PCR product of 1600 bp. The resulting *Salmonella* strain MvP320 was examined for the presence of the resistance cassette within the *ssrB* gene by the use of Southern hybridization analysis of total DNA of exconjugants.

Please replace the paragraph bridging pages 74 and 75 with the following paragraph:

A deletion of 407 codons between codon 44 and 451 of *ssrB* was generated. Plasmid p2-2 was digested by *Bam*HI and *Kpn*I, a fragment of 3.7kb was recovered and subcloned in pBluescript KS+ to generate p2-50. Plasmid p2-50 was linearized by digestion with *Pst*I, which cuts once within the subcloned fragment of the *ssrA* gene. Primers *ssrA*-del-1 (5'- GGT CTG CAG GAT TTT TCA CGC ATC GCG TC -3') (SEQ ID NO:68) and *ssrB*-del-2 (5'- GGT CTG CAG AAC CAT TGA TAT ATA AGC TGC -3') (SEQ ID NO:69) were designed to introduce *Pst*I sites. PCR was performed using linearized p2-50 as template DNA. The TaqPlus polymerase (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x TaqPlus Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p2-50), 250 ng of each primer and 5 U of TaqPlus DNA polymerase. PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for

6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Pst*I, self-ligated, and the ligation mixture was used to transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 µg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *ssrA* locus was performed by PCR using primers *ssrA*-For (5'- AAG GAA TTC AAC AGG CAA CTG GAG G-3') (SEQ ID NO:64) and *ssrA*-Rev (5- CTG CCC TCG CGA AAA TTA AGA TAA TA -3') (SEQ ID NO:65). Amplification of DNA from clones containing the wild-type *ssrA* allele resulted in a PCR product of 2800 bp, use of DNA from clones harbouring a *ssrA* allele with an internal deletion resulted in a PCR product of 1580 bp. The integrity of clones harbouring the *ssrA* deletion was further confirmed by Southernanalysis of the *ssrA* locus. Finally, the *ssrA* locus

containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP340.

Please replace the paragraph on page 78, lines 11-14, with the following paragraph:

Fig. 2a. Alignment of the deduced SseB amino acid sequence (SEQ ID NO:5) to EspA of EPEC (SEQ ID NO:40) (Elliot *et al.*, 1998). The ClustalW algorithm of the MacVector 6.0 program was used to construct the alignments. Similar amino acid residues are boxed, identical residues are boxed and shaded.

Please replace the paragraph on page 78, lines 16-22, with the following paragraph:

Fig. 2b. Alignment of the deduced SseC amino acid sequence (SEQ ID NO:8) to EspD of EPEC (SEQ ID NO:41) (Elliot *et al.*, 1998), YopB of *Yersinia enterocolitica* (SEQ ID NO:42) (Hakansson *et al.*, 1993), and PepB of *Pseudomonas aeruinosa* (SEQ ID NO:43) (Hauser *et al.*, 1998). The ClustalW algorithm of the MacVector 6.0 program was used to construct the alignments. Positions where at least three amino acid residues are similar are boxed, where at least three residues are identical are boxed and shaded.

Please replace the paragraph on page 82, lines 20-22, with the following paragraph:

Fig.21A shows the genomic sequence of a region of the SPI2 locus from Salmonella comprising the complete sequences of the genes ssaE to ssaI and partial sequences of ssaD and ssaJ (cf. Fig.12) (SEQ ID NO:1).

Please replace the paragraph on page 82, lines 24-25, with the following paragraph:

Fig.21B shows the nucleotide sequence of a region of the SPI2 locus from Salmonella comprising the coding sequences for ssrA and ssrB (SEQ ID NO:2).

Please replace the paragraph on page 82, lines 27-28, with the following paragraph:

Figs.22A-Q each show the nucleotide sequence of the respective gene indicated (SEQ ID NOS:3, 5, 7, 9, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36).

Please replace the paragraph on page 82, lines 30-31, with the following paragraph:

Figs.23A-Q each show the amino acid sequence of the respective polypeptide indicated (SEQ ID NOS:4, 6, 8, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, and 37).

Please replace the paragraph on page 83, lines 1-2, with the following paragraph:

Figs.24A,B each show a nucleotide sequence comprising an in vivo inducible promoter (SEQ ID NO:38 and SEQ ID NO: 39).